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Constituents of the Seed of *Malva verticillata*. II.[1] Characterization of Two Novel Neutral Polysaccharides

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Two novel minor polysaccharides, designated as MVS-IIA and MVS-IIG, were isolated from the seeds of *Malva verticillata* L. They were each homogeneous on electrophoresis and gel chromatography, and their molecular weights were estimated to be 57000 and 10400, respectively. MVS-IIA is composed of L-arabinose : D-galactose : D-mannose in the molar ratio of 14 : 28 : 1, and MVS-IIG is composed of D-glucose : D-galactose : D-mannose in the molar ratio of 10 : 1 : 1. Methylation analysis, ^{13}C-nuclear magnetic resonance, and Smith degradation studies enabled elucidation of their structural features.

Keywords——*Malva verticillata*; seed; polysaccharide; MVS-IIA; MVS-IIG; mannoarabino-3,6-galactan; mannogalactoglucan; structural feature

Previously, we isolated and elucidated structural features of the major neutral polysaccharide, designated as MVS-I, from the seed of *Malva verticillata* L. (Malvaceae).[1] For the isolation and purification of MVS-I, affinity chromatography with Con A-Sepharose was used. Now we report the isolation of two novel minor polysaccharides from the fraction which interacts with concanavalin A. Their structural features have been deduced from the results of chemical and physicochemical characterization.

The extraction of polysaccharides with hot water followed by ethanol precipitation and chromatography with diethylaminoethyl (DEAE)-Sephadex A-25 has been described previously.[1] Neutral polysaccharides were obtained from the eluate with water, and applied to a column of Con A-Sepharose after purification by gel chromatography with Sephadex G-25. MVS-I was not retained in the column, and after elution with a phosphate buffer, a new fraction was obtained from the eluate with a phosphate buffer containing methyl-α-D-mannopyranoside. The eluate was dialyzed and purified by gel chromatography on Sephadex G-25. The eluate from this column was concentrated and applied to a column of Cellulofine GCL-2000 m. Two fractions (frs. IIA and IIB) were obtained from the eluate with a phosphate buffer containing sodium chloride. Each of them was purified by gel chromatography with Sephadex G-25 (Chart 1).

The polysaccharides from frs. IIA and IIB are designated as MVS-IIA and MVS-IIG. Each polysaccharide was homogeneous on gel chromatography and glass-fiber paper electrophoresis. MVS-IIA had $[\alpha]_D^{24}$ −42.7° (H_2O, $c=0.37$), and MVS-IIG had $[\alpha]_D^{24}$ +110.8° (H_2O, $c=0.22$). Gel chromatography with standard pullulans gave values of 57000 and 10400 for the molecular weights of MVS-IIA and MVS-IIG, respectively.

Quantitative analysis showed that MVS-IIA contained 30.3% L-arabinose, 67.4% D-galactose, and 2.4% D-mannose, and that their molar ratio was 14 : 28 : 1. MVS-IIG contained 83.3% D-glucose, 8.2% D-galactose, and 7.9% D-mannose, and their molar ratio was approximately 10 : 1 : 1. The nuclear magnetic resonance (NMR) spectra and infrared (IR) spectra of MVS-IIA and of MVS-IIG showed no acetyl signal or absorption. No nitrogen was found in either of the polysaccharides.

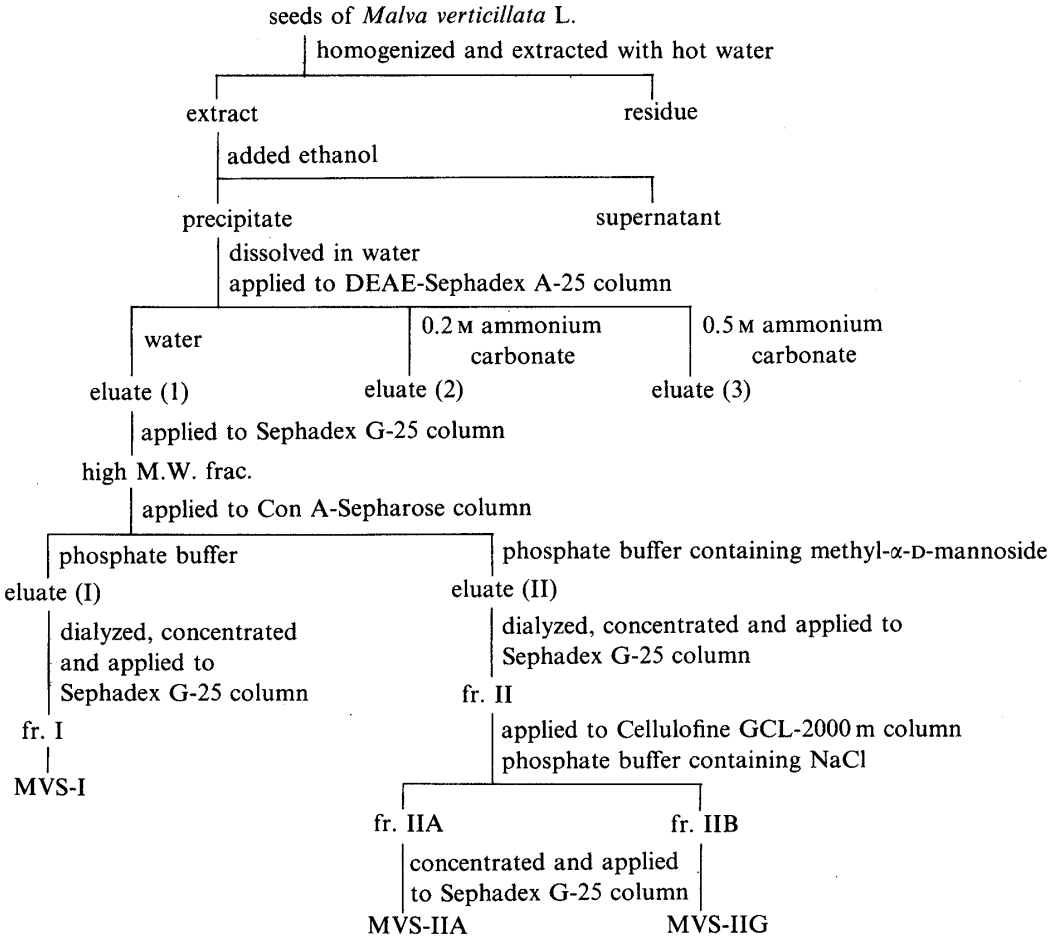

Chart 1. Isolation of Neutral Polysaccharides

Methylation of the polysaccharides was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[2] The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. Gas-liquid chromatography (GLC)-mass spectrometry (MS)[3] revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, and 2,3,4,6-tetra-*O*-methyl-D-mannose as the products in a molar ratio of 6:8:3:15:10:1 from MVS-IIA. MVS-IIG gave derivatives of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose, and 2,3,4,6-tetra-*O*-methyl-D-mannose as the products in a molar ratio of 1:7:2:1:1.

The carbon 13 nuclear magnetic resonance (^{13}C-NMR) of MVS-IIA showed four signals due to anomeric carbons at δ 111.916, 110.135, 105.872, and 102.016 ppm. The first two signals were assigned to the anomeric carbons of α-L-arabinofuranose,[1,4] and the signals at 105.872 and 102.016 ppm to the anomeric carbons of β-D-galactopyranose and α-D-mannopyranose residues, respectively.[5,6] The glycosidic configuration of D-mannose units in MVS-IIA is also supported by the lectin-binding specificity of the polysaccharide. In the ^{13}C-NMR spectrum of MVS-IIG, three anomeric carbon signals were observed at 102.338, 102.198, and 101.259 ppm. These were assigned to the anomeric carbons of α-D-mannopyranose, α-D-glucopyranose, and α-D-galactopyranose.[5,6] These results suggested that the minimal repeating unit of MVS-IIA is composed of six kinds of component sugar units as shown in Chart 2, and that the repeating unit of MVS-IIG is composed of five kinds

			↓ 6
(six)[a]	α-L-Ara*f* 1→	(ten)[a]	→3 β-D-Gal*p* 1→
(eight)[a]	→5 α-L-Ara*f* 1→	(fifteen)[a]	→3 β-D-Gal*p* 1→
(one)[a]	α-D-Man*p* 1→	(three)[a]	β-D-Gal*p* 1→

Chart 2. Component Sugar Residues of the Minimal Repeating Unit in the Structure of MVS-IIA

a) Number of residues.
Ara*f*, arabinofuranose; Man*p*, mannopyranose; Gal*p*, galactopyranose.

	↓ 6		
(two)[a]	→4 α-D-Glc*p* 1→	(one)[a]	→3 α-D-Gal*p* 1→
(seven)[a]	→4 α-D-Glc*p* 1→	(one)[a]	α-D-Man*p* 1→
(one)[a]	α-D-Glc*p* 1→		

Chart 3. Component Sugar Residues of the Minimal Repeating Unit in the Structure of MVS-IIG

a) Number of residues.
Glc*p*, glucopyranose; Gal*p*, galactopyranose; Man*p*, mannopyranose.

[→3 β-D-Gal*p* 1→]$_a$[→6 β-D-Gal*p* 1→]$_b$
3
↑
side chains A, B, C

Chart 4. Structural Units of MVS-IIA

Side chain A, α-L-Ara*f* 1→[5 α-L-Ara*f* 1→]$_c$; side chain B, β-D-Gal*p* 1→[5 α-L-Ara*f* 1→]$_d$; side chain C, α-D-Man*p* 1→[5 α-L-Ara*f* 1→]$_e$.
a : b = 3 : 2, a + b = 25, 6c + 3d + e = 8. side chain A : side chain B : side chain C = 6 : 3 : 1.

of components as shown in Chart 3.

The controlled Smith degradation product was obtained from MVS-IIA by periodate oxidation and reduction followed by mild hydrolysis.[7] The sole component sugar of the product was D-galactose, and the approximate molecular weight of the product was 40000. Thus, Smith degradation of MVS-IIA resulted in complete elimination of the sugar residues located at side chains in the molecule. Methylation of the controlled Smith degradation product followed by hydrolysis, reduction, acetylation, and analysis by GLC-MS revealed 2,4,6-tri-*O*-methyl-D-galactitol acetate and 2,3,4-tri-*O*-methyl-D-galactitol acetate as the resulting products in a molar ratio of 3 : 2. Thus, the polymer is composed of β-1→3-linked D-galactopyranosyl residues and β-1→6-linked D-galactopyranosyl backbone units. The latter units were apparently derived from the D-galactopyranosyl units having branching points at position 3 to which side chains had been attached in the original polysaccharide.

Based on the accumulated evidence described here, it may be concluded that MVS-IIA contains the units shown in Chart 4. However, the sequence of β-1,3-linked and β-1,6-linked D-galactopyranosyl residues is not yet known.

Structural studies on MVS-IIA provided evidence that the polysaccharide was essentially one of the type II arabino-3,6-galactans as defined in Aspinall's classification.[8] The type II arabinogalactans are widely distributed in various plant sources. The majority have a backbone chain composed of 1,3-linked β-D-galactopyranose residues with 1,6-branched units, and some of them have a partially 1,6-linked β-D-galactopyranosyl backbone with 1,3-branched units. In general, they possess β-D-galactopyranosyl side chains binding to the other sugar residues at their 3 and/or 6 positions.[9] In MVS-IIA, most of the side chains are

characteristically 1,5-linked α-L-arabinofuranosyl units, and the polysaccharide possesses three branch-terminal β-D-galactopyranose residues per twenty-eight D-galactose units. The presence of α-D-mannopyranosyl terminal residues is especially unique; as far as we are aware, this is the first description of α-D-mannose residues in such a terminal position on arabinogalactans.

MVS-IIG is apparently an amylopectin-type glucan. However, in contrast to amylopectin, the average length of unit chains in it is very short. In addition, the polysaccharide possesses a novel side chain containing α-D-mannopyranose units. The presence of 1,3-linked α-D-galactopyranosyl residues is an additional unique feature of MVS-IIG as a glucan of this type.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotations were measured with a JASCO DIP-140 automatic polarimeter. NMR spectra were recorded on a JEOL JNM-GX 270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30 °C. IR spectra were measured with a JASCO IRA-2 infrared spectrophotometer. GLC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GLC-MS was performed with a JEOL JMS-GX 303 mass spectrometer.

Isolation of Polysaccharides——The material was imported from China as described previously.[1] The seeds (200 g) were homogenized and extracted with hot water (2000 ml) under stirring for 1 h in a boiling water bath. After suction filtration, the filtrate was poured into two volumes of ethanol. After centrifugation and drying, the precipitate was dissolved in water (200 ml). The solution was applied to a column (5 × 78 cm) of DEAE-Sephadex A-25. DEAE-Sephadex A-25 was pretreated as described in a previous report.[10] The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[11] The eluates obtained from tubes 9 to 49 were combined and concentrated, and half of the concentrate was applied to a column (5 × 78 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 27 to 32 were combined, concentrated and applied to a column (1.5 × 38 cm) of Con A-Sepharose (Pharmacia Co.). The column was equilibrated with 0.067 M phosphate buffer (pH 7) containing 0.15 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and kept at 4 °C. After elution with the same buffer (140 ml), the column was eluted with 0.01 M methyl-α-D-mannopyranoside in the same buffer solution. Fractions of 10 ml were collected, and the eluates obtained from tubes 7 to 21 were combined, dialyzed against distilled water and concentrated. The solution was applied to a column (5 × 81 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 28 to 31 were combined and concentrated. The solution was applied to a column (2.6 × 94.5 cm) of Cellulofine GCL-2000 m (Seikagaku Kōgyo Co.). The column was equilibrated with 0.05 M phosphate buffer (pH 7.5) containing 0.1 M NaCl. The column was eluted with the same buffer, and fractions of 10 ml were collected. The eluates obtained from tubes 31 to 37 gave fr. IIA, and the eluates obtained from tubes 42 to 46 gave fr. IIB. Each fraction was concentrated and applied to a column (5 × 84.5 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 30 to 32 were combined, concentrated and lyophilized. MVS-IIA and MVS-IIG were obtained as white powders (yield, 7.5 mg in MVS-IIA and 3.0 mg in MVS-IIG from 200 g of seeds).

Molecular Weight——This was calculated from the results of gel chromatography as described in a previous report.[1] Standard pullulans having known molecular weights were run on the column to obtain a calibration curve.

Glass-Fiber Paper Electrophoresis——This was performed as described in a previous report[12] on Whatman GF/C glass-fiber paper at 570 V for 50 min with 0.025 M borax–0.1 N sodium hydroxide (10 : 1, pH 9.46). MVS-IIA and MVS-IIG each gave a single spot at distances of 11.5 and 12.0 cm, respectively, from the origin toward the anode.

Qualitative Analysis of Components——Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were carried out as described in a previous report.[10] The configurations of component sugars were proved by GLC of the trimethylsilylated α-methylbenzylaminoalditol derivatives.[13]

Determination of Components——Each sample was hydrolyzed, reduced and acetylated as described in a previous report.[14] The derivatives were analyzed by GLC as described in a preceding report.[1]

Methylation——This was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.[15] The methylation was repeated three times under the same conditions. Yields were 5.1 mg from 5.7 mg of MVS-IIA, and 4.2 mg from 5.7 mg of MVS-IIG.

Analysis of the Methylated Products——The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[16] GLC-MS of partially methylated alditol acetates was performed with a fused silica capillary column (0.32 mm i.d. × 30 m) of SP 2330 (Supelco Co.) and with a

TABLE I. Relative Retention Times on GLC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a)]	Main fragments (*m/z*)
1,4-Ac-2,3,5-Me-L-Arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac-2,3-Me-L-Arabinitol	1.12	43, 87, 101, 117, 129, 189
1,5-Ac-2,3,4,6-Me-D-Mannitol	0.98	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,5-Ac-2,3,4,6-Me-D-Galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac-2,4,6-Me-D-Galactitol	1.29	43, 45, 87, 101, 117, 129, 161
1,5,6-Ac-2,3,4-Me-D-Galactitol	1.58	43, 87, 99, 101, 117, 129, 161, 189
1,3,5,6-Ac-2,4-Me-D-Galactitol	2.00	43, 87, 117, 129, 189
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Glucitol	1.45	43, 45, 87, 99, 101, 113, 117, 233
1,4,5,6-Ac-2,3-Me-D-Glucitol	1.92	43, 101, 117, 261

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac=acetyl; Me=methyl (*e.g.*, 1,4-Ac-2,3,5-Me-=1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow rate of 1 ml per min. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GLC and their main fragments in the mass spectra are listed in Table I. A part of the methylation product of MVS-IIG was methanolyzed and the product was analyzed by GLC in the manner described in a previous report[17]; methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside and methyl 2,3,4,6-tetra-*O*-methyl-D-mannoside were identified.

Smith Degradation of MVS-IIA——The sample (16.4 mg) was oxidized with 0.05 M sodium metaperiodate (8 ml) at 7 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[18] The oxidation was completed after 3 d, and the maximal value of consumption was 0.54 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (0.08 ml) at 7 °C for 1 h and sodium borohydride (80 mg) at 7 °C for 16 h, then adjusted to pH 5 by addition of acetic acid. The solution was dialyzed against distilled water. The non-dialyzable fraction was concentrated and applied to a column (2.6 × 86 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 18 to 20 were combined, concentrated and lyophilized. Yield, 14.4 mg. This product was dissolved in 0.5 N sulfuric acid (1.5 ml), and, after standing at room temperature for 16 h, the solution was neutralized with barium carbonate and filtered. The filtrate was concentrated and applied to a column (2.6 × 86 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 19 to 21 were combined, concentrated and lyophilized.

Methylation Analysis of the Smith Degradation Product——This was performed in the same manner and under the same conditions as those described above. The relative retention times of the products in GLC with a capillary column of SP 2330 and their main mass fragments in the mass spectra are also shown in Table I.

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